

Development and Properties of a Wax Ester Hydrolase in the Cotyledons of Jojoba Seedlings¹

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ABSTRACT

The activity of a wax ester hydrolase in the cotyledons of jojoba (*Simmondsia chinensis*) seedlings increased drastically during germination, parallel to the development of the gluconeogenic process. The enzyme at its peak of development was obtained in association with the wax body membrane, and its properties were studied. It had an optimal activity at alkaline pH (8.5-9). The apparent K_m value for N-methylindoxylmyristate was 93 μ M. It was stable at 40 C for 30 min but was inactivated at higher temperature. Various divalent cations and ethylenediaminetetraacetate had little effect on the activity. *p*-Chloromercuribenzoate was a strong inhibitor of the enzyme activity, and its effect was reversed by subsequent addition of dithiothreitol. It had a broad substrate specificity with highest activities on monoglycerides, wax esters, and the native substrate (jojoba wax).

The seeds of jojoba contain 50 to 60% of their fresh wt as intracellular wax esters which are present in the cotyledons (11). In a previous report (4), we established that the wax serves as a food reserve. During germination, the wax is metabolized to sugars for the growth of the seedlings by a metabolic pathway similar to that in triglyceride containing fatty seedlings. Using an artificial substrate, N-methylindoxylmyristate, we detected the activity of a lipase³ that was associated with the membrane of the wax body. In the present paper, we report the development and properties of this lipase in the jojoba seedlings.

MATERIALS AND METHODS

Seeds of jojoba (*Simmondsia chinensis* [Link] Schneider) were dusted with Phaltan (Chevron Chem. Co., Richmond, Calif.) and allowed to germinate as described (4). In the preparation of total extracts for the determination of lipase and catalase activities, the cotyledons of various ages were homogenized in grinding medium (4) with a VirTis homogenizer at medium speed. In the preparation of wax bodies, the cotyledons were first chopped into small pieces in grinding medium with an onion chopper and then ground with a mortar and pestle. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 10,000g for 30 min. The wax body layer on top of the centrifuge tube was removed by a spatula and resuspended in grinding medium. Centrifugation and resuspension were repeated twice. The final resuspended wax bodies were extracted five times with diethyl

ether to remove the wax. The trace amount of diethyl ether remaining was evaporated under a stream of N₂.

Catalase activity (2) and protein (3) were assayed spectrophotometrically as described. Lipase activity was assayed by either fluorometric or colorimetric method. In the fluorometric method, a reaction mixture of 4 ml containing 0.1 M Tris-HCl buffer (pH 9), 2 mM DTT, and 0.83 mM N-methylindoxylmyristate in ethylene glycol monomethyl ether (0.1 ml) was used (5). A modified colorimetric assay (5) was used to measure the enzyme activities when various lipids were tested as potential substrates. In this method, the fatty acids produced were converted to copper soaps and measured using sodium dithiocarbamate. The reaction mixture contained in a final volume of 1 ml: 100 mM Tris-HCl (pH 9), 5 mM DTT, 10 mM substrate, and enzyme. Substrates were emulsified in 5% gum acacia for 1 min at low speed with a Bronwill Biosonik IV ultrasonic generator fitted with a microprobe. For each substrate, two enzyme concentrations were used and the reaction was stopped at several time intervals to ensure that proper kinetic data were obtained. All assays were carried out at room temperature (24 C).

1,3-Dipalmitin and tripalmitin were obtained from Sigma, N-methylindoxylmyristate from ICN Pharmaceuticals, Inc. (Cleveland), jojoba oil and hydrogenated jojoba oil from the Office of Arid Lands Studies, University of Arizona, and all other lipids from Nu Check (Elysian, Minn.).

RESULTS

The total activity of lipase in the cotyledons of jojoba seedlings increased drastically during the first 15 days of germination; thereafter it leveled off (Fig. 1). Its development pattern followed closely that of catalase activity which has been shown to be a marker of the gluconeogenic process (4).

We reported earlier (4) that the lipase in the cotyledons of 20-day-old seedlings was associated with the membrane of the wax bodies. In the present study, the membranes of the wax bodies were prepared from isolated wax bodies by removing the wax with diethyl ether. The membrane preparation had a specific activity of 28.6 nmol/min · mg of protein on N-methylindoxylmyristate, and represented a 14% recovery and 8-fold purification. An effort to remove the enzyme from the membrane using osmotic shock, 1 M KCl, or 0.05% deoxycholate was unsuccessful. We then used the membrane-associated enzyme to study its properties.

Using an artificial substrate, N-methylindoxylmyristate, the enzyme exerted optimal activity at pH between 8.5 and 9 (Fig. 2), and had an apparent K_m value of 93 μ M (Fig. 3). It was stable at 40 C for 30 min but was inactivated at higher temperature (Fig. 4). Various divalent and monovalent cations (1 mM of CaCl₂, MgCl₂, NaCl, or KCl), EDTA (1 mM), and DTT (5 mM) exerted little effect on the enzyme activity. *p*-Chloromercuribenzoate at 0.1 mM inhibited 81% of the enzyme activity and its effect was reversed by subsequent addition of 5 mM DTT.

The substrate specificity of the enzyme is shown in Table I. In general, monoglycerides gave the highest activities, with a gradual

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³ For convenience, we have referred to the wax ester hydrolase of jojoba as a lipase (EC 3.1.1.3), although it does not fit the strictest definition (1).

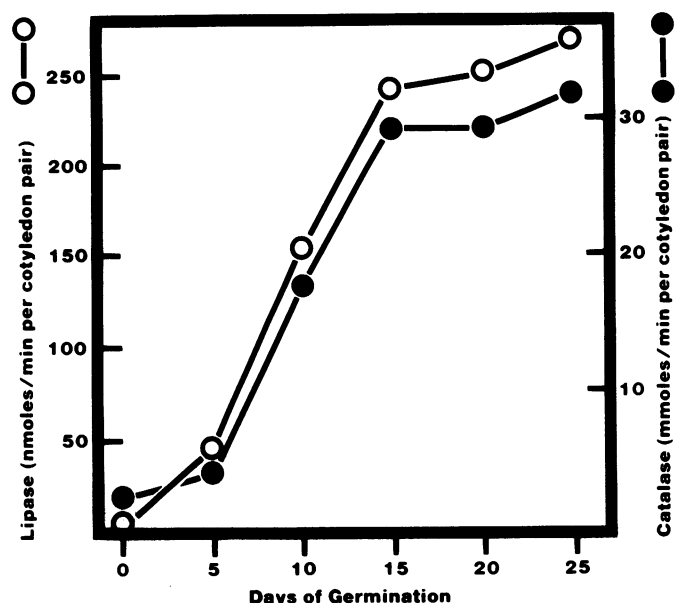


FIG. 1. Changes in the total activities of lipase and catalase in the cotyledons of jojoba seedlings during germination.

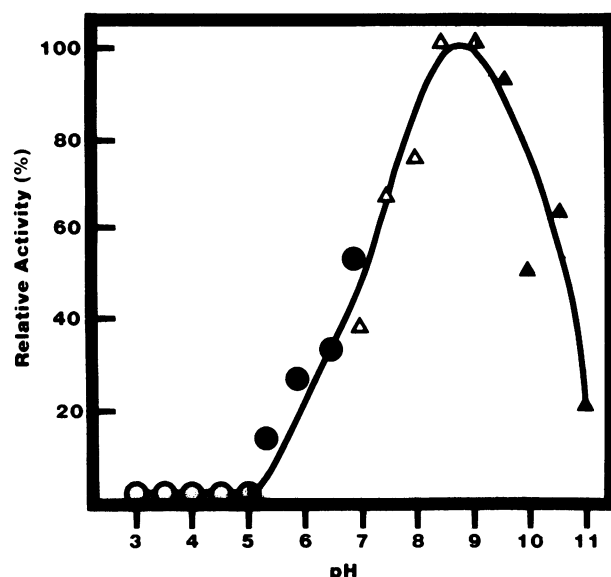


FIG. 2. Effect of pH on the activity of the partially purified jojoba lipase. O: citrate-NaOH buffer; ●: cacodylate-NaOH buffer; △: Tris-HCl buffer; ▲: CHES-NaOH buffer.

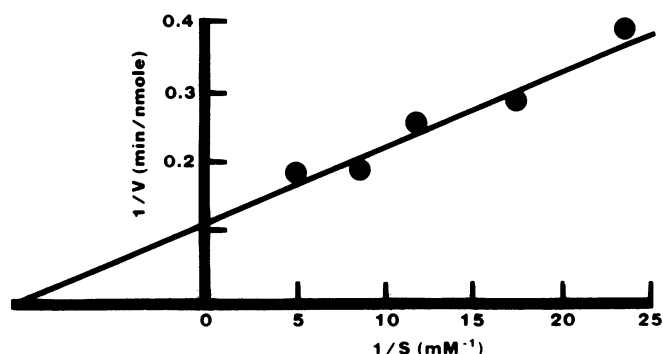


FIG. 3. Double reciprocal plot of the activity of the partially purified jojoba lipase as a function of the concentrations of N-methylindoxylmyristate.

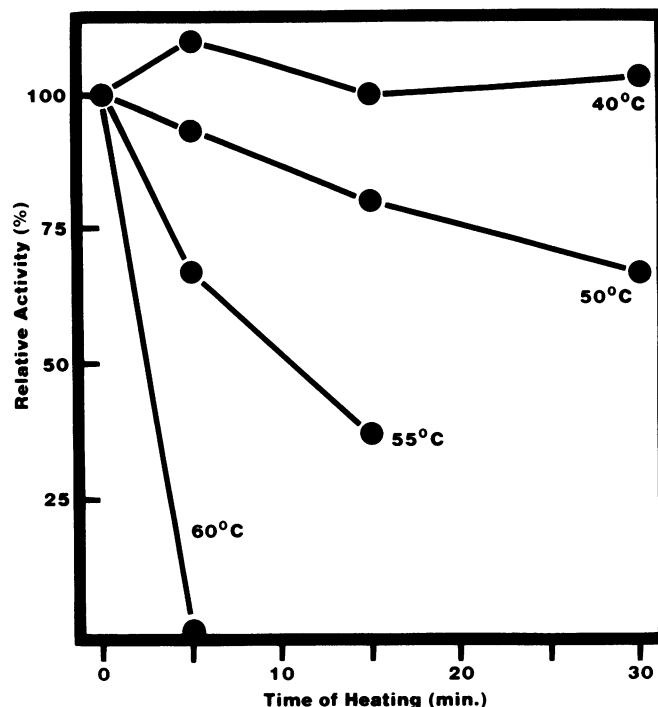


FIG. 4. Heat stability of the partially purified jojoba lipase.

Table I.

Hydrolysis of Various Substrates by the Partially Purified Jojoba Lipase.

The activity on monopalmitin (3.13 nmol/min.ml) was used as a standard in each experiment and the activities on all other substrates are expressed as relative to it. The data were obtained from numerous experiments. All the monoglycerides were the α forms.

Substrates	Relative Activity
Monolaurin	213
Monomyristin	150
Monopalmitin	100
Monostearin	58
Monoarachidin	40
Myristyl-myristate	100
Palmityl-palmitate	67
Stearyl-stearate	84
Arachidyl-arachidate	47
Jojoba oil	54
Hydrogenated jojoba oil	42
1,3-dipalmitin	26
Tripalmitin	33
Triolein	20

decrease in activities as the chain length of the fatty acids increased. The four commercially prepared wax esters were hydrolyzed at slightly lower rates than those of the monoglycerides. The wax ester with the shortest fatty components, myristyl-myristate, was hydrolyzed at a rate similar to that of monopalmitin. The activity of the enzyme on its natural substrate, jojoba wax, was 54% of that observed for monopalmitin. Jojoba wax is comprised of wax esters of monounsaturated fatty acids and fatty alcohols with mostly 18, 20, or 22 carbons in each fatty component (11). The activity of the enzyme on hydrogenated (saturated) jojoba wax was slightly lower. The enzyme exerted low activities on diglycerides and triglycerides.

DISCUSSION

In recent years, only a few lipases of higher plants have been studied. Each of these studies has dealt with lipases from seeds which contain triglycerides as food reserves. The three well characterized lipases from fatty seeds are castor bean acid lipase (7), castor bean alkaline lipase (5), and peanut alkaline lipase (9). Although no other higher plants are known to store wax esters

as food reserves, many marine invertebrates and vertebrates store and metabolize them (6). An alkaline lipase from the surf clam hydrolyzes palmityl-oleate at one-sixth the rate of triolein hydrolysis, while hog pancreatic lipase hydrolyzes the same wax at one-twentieth the rate of triolein hydrolysis (8). The alkaline lipase of jojoba hydrolyzes jojoba wax almost three times faster than triolein, and therefore seems to be better suited for wax ester hydrolysis than either of these animal lipases.

The alkaline lipase of jojoba shares two features in common with all other plant lipases which have been studied. First, inhibition of enzyme activity by *p*-chloromercuribenzoate was also observed in the two lipases of castor bean (5, 7), peanut alkaline lipase (9), and wheat germ lipase (10). Second, other lipases are similarly membrane-associated. The castor bean acid lipase is localized in the membranes of the spherosomes (7), while the castor bean alkaline lipase is tightly bound to the membranes of the glyoxysomes (5). Alkaline lipases in glyoxysomes of fatty seedlings are also associated with the organelle membranes (2). The association of these lipases with organelle membranes is probably essential for the proper orientation of the enzymes to receive substrates and to deliver products inside the cells. Jojoba lipase exhibits very little activity in the dry seeds and develops peak activity only after 15 to 20 days of germination. The developmental patterns of the two castor bean lipases (5) suggest that they are likely synthesized together with the membrane during organelle biogenesis. Since the jojoba wax body membranes have

already been formed before germination, appearance of the lipase activity during germination may be due either to an incorporation of newly synthesized enzymes originated from the cytoplasm or to an activation of a preexisting enzyme in the membrane.

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